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An *in vivo* Experimental System to Study Sugar Phloem Unloading in Ripening Grape Berries During Water Deficiency Stress

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An *in vivo* experimental system—called the 'berry-cup' technique—was developed to study sugar phloem unloading and the accumulation of sugar in ripening grape berries. The berry-cup system consists of a single peeled grape berry immersed in a buffer solution in a cup prepared from a polypropylene syringe. A small cross-incision (2 mm in length) is made on the stylar remnant of a berry during its ripening phase, the skin of the berry then being easily peeled off, exposing the dorsal vascular bundles without damaging either these or the pulp tissue of the berry. The sites of sugar phloem unloading are thus made directly accessible and may be regulated by the buffer solution. In addition, the unloaded photoassimilates are easily transported into the buffer solution in the berry-cup. With the berry-cup technique, it takes 60 min to purge the sugar already present in the apoplast, after which the amount of sugar in the buffer solution is a direct measure of the sugar unloading from the grape berry phloem. The optimum times for sampling were 20 or 30 min, depending on the type of experiment. Sugar phloem unloading was significantly inhibited by the inclusion of either 7·5 mM NaF or 2·5 mM PCMB in the buffer solution. This study indicates that sugar phloem unloading in ripening grape berries is via the apoplastic network and that the process requires the input of energy. The system was shown to be an appropriate experimental system with which to study sugar phloem unloading in ripening grape berries, and was applied successfully to the study of berry sugar unloaded from grapevines subjected to water stress. The results showed that water deficiency inhibits sugar unloading in grape berries.

Key words: Sugar phloem unloading, 'berry-cup' technique, ripening grape berry, inhibitors, water deficiency.

INTRODUCTION

In the last two decades, following an initial surge of interest in the mechanism of phloem loading in leaves, a number of scientists turned their attention to sink regions of the plant and to the study of the pathway and mechanisms of phloem unloading. It is now well accepted that the sink regions of plants exert considerable influence in determining the pattern of photoassimilate distribution in plants (Ho, 1988; Patrick, 1990, 1997). Phloem unloading has been defined as the series of transport steps that solutes make from the sieve element lumen to sites of utilization in the recipient sink cells (Oparka, 1990). It is necessary to investigate the mechanisms of phloem unloading so that the effect of environmental factors on sugar accumulation in sink organs can be determined.

Following long-distance transport in the phloem, the exit of photoassimilates from the sieve elements is the first step in a complex series of short-distance steps within the sink organs (Oparka, 1990). Some techniques have been described for the specific study of phloem unloading. For example, the 'empty seed coat' or 'empty-ovule' method was used for the study of phloem unloading in seed coats (Thorne and Rainbird, 1983; Wolswinkel and Ammerlaan, 1983; Gifford and Thorne, 1986). The 'unloading trap technique' was used for the study of phloem unloading in growing potato tubers (Oparka and Prior, 1987) and apples (Lü *et al*., 1999). These methods have the drawbacks of amputating the phloem and injuring the sink organ tissue, and make it difficult to find a suitable position to approach the phloem unloading point. The 'tissue disk technique' was used to study photoassimilate unloading, and the accumulation and metabolism of sugar in tomatoes (Damon *et al*., 1988), strawberries (Ofosu-Anim and Yamaki, 1994) and grape berries (Findlay *et al*., 1987). This method can also injure the phloem and surrounding tissue and does not produce reproducible results.

An isotopic tracer technique has also been used to study phloem unloading (Thorne and Rainbird, 1983; Wolswinkel and Ammerlaan, 1983; Xia, 1999). Not only can this method alter the growing environment of the plant, but the technique is cumbersome, costly and requires the availability of highly skilled personnel. Therefore, we considered it important to find a new technique to help quantify sugar phloem unloading in grape (*Vitis vinifera* L.) berries.

Grape berries exhibit a double sigmoid pattern of development, with two distinct phases of growth separated by a lag phase (Coombe, 1992; Robinson and Davies, 2000). The ripening phase in the maturation of grape berries occurs during the second growth period and results from the expansion, without multiplication, of pericarp cells (Ojeda *et al*., 2001). Veraison after the lag phase is defined as the beginning of berry ripening and sugar accumulation. This

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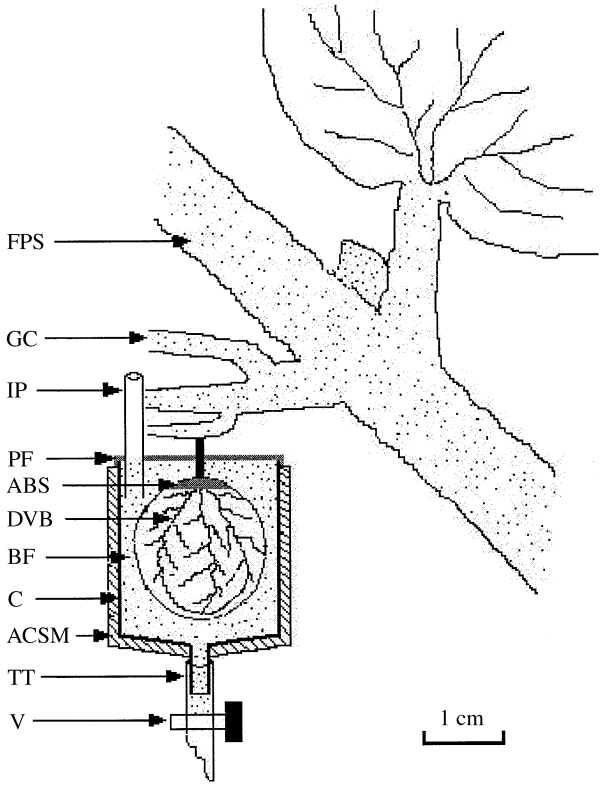

FIG. 1. Experimental arrangement of the 'berry-cup' system for the study of sugar phloem unloading. ACSM, Aluminium-coated sponge material; BF, buffer solution; C, cup; DVB, dorsal vascular bundle; FPS, fruiting primary stem; GC, grape cluster; IP, injection pipe; V, valve; PF, Parafilm; RBS, remaining berry skin; TT, Teflon tube.

stage is characterized by berry softening and the accumulation of anthocyanins in red grape varieties (Coombe, 1992). Sugar accumulation occurs during the ripening phase and begins immediately after softening.

In this paper, a novel method is described which uses a berry-cup in an *in vivo* experimental system to study sugar phloem unloading in ripening grape berries. The method can also be used to study the effect of various internal and external factors on sugar accumulation in grape berries.

MATERIALS AND METHODS

Plant material and growth conditions

All experiments were performed on 12-year-old Syrah vines (*Vitis vinifera* L.), grafted on Fercal rootstocks, trained on lyre (the official name for a type of grapevine training system), planted in pots of 70 l in the Ecotron experimental system (AGRO-Montpellier, France). The rows of vines were arranged in a north–south orientation. The horticultural substratum was a mixture of Perlite and sand (9 : 1 by volume). Fertilization and irrigation were assured by a drip-irrigation system. Plant water status was controlled by the predawn leaf water potential (Ψ_b) (Scholander *et al.*, 1965; Carbonneau, 1998). The Ψ_b of the control vines was $0 \geqslant \Psi_b \geqslant -0{\cdot}2$ MPa and $-0{\cdot}5 \geqslant \Psi_b \geqslant -0{\cdot}6$ MPa for the water-stressed vines.

Preparation of the berry-cup in vivo *experimental system*

The experiment was carried out between veraison and ripening. Well-developed primary stems with grape clusters were chosen from ten individual vines; one-third of the berries were cut away to facilitate the setting up of the berry-cup. The berry-cups were installed in the centre of the grape clusters and a berry close to the stem was chosen for the berry-cup. The cup parts of the berry-cup configuration were prepared by cutting 20 ml polypropylene syringes to a height of 2 cm from the exit spout of the syringe; the total capacity of the cup was 10 ml. The exit spout of the syringe was connected to a Teflon tube (TT in Fig. 1) fitted with a valve (V in Fig. 1) to permit emptying of the cup. The cup was filled with buffer by means of an injection pipe (IP in Fig. 1). The surface of the cup was covered with an aluminum-coated sponge material (ACSM in Fig. 1) to protect the berry-cup from light and to keep the temperature at 24–30 °C.

A small cross-incision (2 mm in length) was made with a scalpel on the stylar remnant, which is the end link point of the dorsal vascular bundles. The incision was made so as not to damage the pulp tissue. The berry skin was then completely peeled away from the stylar remnant with tweezers and scissors. Different buffer solutions were evaluated in order to determine the buffer that most closely resembles the natural pH and ionic strength of the grape berry. The buffer finally selected consisted of 5 mM 2(*N*-morpholino) ethanesulfonic acid (MES), 2 mM $CaCl_2$, 100 mM D-mannitol and 0·2 % (w/v) polyvinylpyrrolidone (PVP), pH 5·5, otherwise referred to as 'standard MES buffer'. This buffer solution was added into a prepared cup, followed immediately by the peeled berry. The top of the berry-cup was then sealed with Parafilm (PF in Fig. 1). If necessary, the buffer solution could be topped up via the injection pipe (IP in Fig. 1). To change buffer solutions, the valve on the Teflon tube was opened and the entire contents drained into a test-tube. After closing the valve, fresh buffer was introduced via the injection pipe. Buffer solution could be replaced in 30 s maximum.

Determination of the experimental collecting time

To determine how quickly the buffer solution became saturated with sugar, 18 berry-cups were prepared and divided into six groups of three. The buffer solution of the three berry-cups in the first group was drained into test-tubes after 10 min, and after 20, 30, 40, 50 and 60 min for the second to sixth groups, respectively. The 18 samples were then frozen at –80 °C before analysis.

Determination of the efflux kinetics of the sugars in vivo

To determine the efflux kinetics of the sugar in a peeled berry *in vivo*, the buffer solution was replaced every 20 min with fresh buffer solution. Each drained buffer solution constituted one sample. For any given berry-cup, the

maximum number of buffer changes was seven, amounting to a total of seven samples for each berry-cup within an experimental period of 140 min. As three berry-cups were prepared in this experiment, the total number of samples was 21. The samples (i.e. drained buffer solutions) were frozen at –80 °C before analysis. This experiment was repeated three times

Preparation and treatment of metabolic inhibitors

The respiratory metabolic inhibitor, sodium fluoride (NaF) was chosen because of its inhibitory effect on the inhibition of energy production which in turn inhibits sugar phloem unloading (Thorne, 1985; Xia, 1999). The NaF solution was prepared prior to each experiment by adding it to the MES buffer; a concentration of 7·5 mM NaF being used as previously described by Xia (1999). *p*-(chloromercurio) benzoic acid (PCMB), a modifier of cysteine groups, was also chosen, because of its inhibitory effects on sugar transportation, which in turn inhibits apoplastic phloem unloading. The PCMB solution was prepared prior to each experiment by incorporating it into to the MES buffer at a concentration of 2·5 mM. To determine the effect of the inhibitors on sugar phloem unloading, three groups of three berry-cups were used. In the first (control) group, the buffer solution was changed every 30 min over a period of 180 min. In the other two (treatment) groups, the berry-cups were treated in the same way as the controls for 30 min (i.e. 30 min immersion in standard MES buffer solution), followed by immersion for 3 × 30 min in buffer containing either 7·5 mM NaF or 2·5 mM PCMB. For the four succeeding immersions, the samples were treated in the same way as the control group. In both cases (control and treatment groups) sampling was carried out after an apoplastic draining period of 60 min and the samples were frozen at –80 °C before analysis. This experiment was repeated three times.

The variation in the percentage of sugar phloem unloading was calculated using the following expression:

$$\Delta S = \frac{(S_0 - S_n)}{S_0} \times 100\%$$

where ΔS is the change in percentage of sugar phloem unloading; S_0 is the quantity of sugar phloem unloaded during the first 30 min and S_n is the quantity of sugar phloem unloading after n buffer collections.

Sugar analysis

The collected samples were thawed at room temperature (20 °C), and adjusted to 10 ml with distilled water. A 50 µl aliquot was used to determine the concentrations of sucrose, D-glucose and D-fructose by an enzymatic kit method (Boehringer Mannheim, Darmstadt, Germany) using a spectrophotometer.

To follow the berry growth we weighed samples of 50 berries from the population (several repetitions). The quantity of sugar phloem unloading was expressed in terms of weight per gram of fresh berry. We cannot weigh the berries used for the 'berry-cup' so we measured their diameter and weighed berries from the population with a similar diameter (there is a correlation between berry diameter and fresh weight; $R^2 = 0{\cdot}98$).

Effect of water stress on the phloem sugar unloading

The berry-cup technique was used to compare berry sugar unloading in normally hydrated and water stressed grapevines. Grapevine water status was measured in terms of the predawn leaf water potential (Ψ_b) (Scholander *et al*., 1965; Carbonneau, 1998). During phase I and the early parts of phase II (until 54 d after anthesis, DAA), all the grapevines were irrigated normally (Ψ_b of the grapevines was $\Psi_b \cong -0{\cdot}2$ MPa). In late phase II (54 DAA, i.e. 5 d before veraison, DBV), the grapevines were divided into two groups, control grapevines and treated (water-stressed) grapevines, each group consisting of 11 plants. The experiments were carried out weekly during the maturation period of the berries during which the Ψ_b of the control grapevines was determined to be $\Psi_b \cong -0{\cdot}2$ MPa and that of the water-stressed grapevines was $-0{\cdot}5 \geqslant \Psi_b \geqslant -0{\cdot}6$ MPa. By way of an example, the results obtained for an experiment carried out 82 d after anthesis, i.e. 23 d after veraison (DAA) are presented. A kinetic study was carried out over a single day on the same berry-cup at four time intervals (0900 h, 1200 h, 1500 h and 1800 h). The buffer was replaced every 30 min, and with each buffer change, the berry-cups were immediately refilled with fresh buffer solution to prevent desiccation of the berry. The apoplastic sugar was purged with the buffer solution for 60 min before the first experiment at 0900 h. The 24 samples were then frozen at –80 °C before analysis.

RESULTS AND DISCUSSION

Experimental system to study sugar phloem unloading in ripening grape berries

To investigate sugar phloem unloading in grape berries reliably, it is essential to establish an experimental system that does not cause injury to either the phloem or the pulp. It is also important to reduce the distance between the phloem and the buffer solution (Thorne, 1985; Ho, 1988).

In mature berries, the skin can be removed readily, thus exposing the dorsal vascular bundles. The sites of sugar phloem unloading are therefore easily accessible by the buffer, which may modulate the flow of sugar in the phloem. Furthermore, the unloaded photoassimilates can easily be transported into the buffer solution in the berry-cup. The berry-cup design (Fig. 1) is such that the operations of collecting and changing the buffer solution are accomplished easily and quickly.

Determination of the experimental collecting time

The accumulation of sugar in the buffer solution increased over 50 min, after which the solution became saturated, as evidenced by the levelling off of the sugar

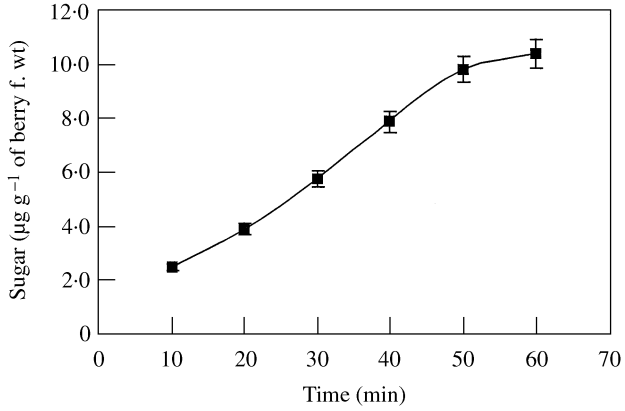

FIG. 2. Buffer sugar content as a function of sampling time. The buffer solution of the berry-cup becomes saturated after 50 min. The results shown are the mean values (n = 3 replicate berry-cups) of sugar efflux (μg g^{-1} berry) from the peeled berry into the buffer solution.

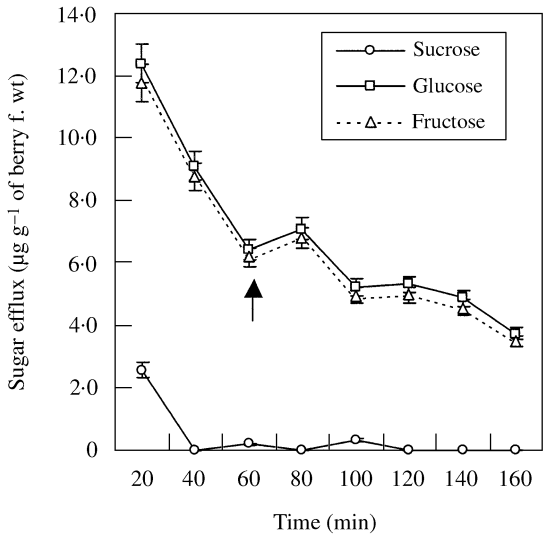

FIG. 3. Efflux of sucrose, glucose and fructose from a peeled berry over 160 min. For the first 60 min (arrow) the decline in sugar efflux corresponds to apoplastic purging. Thereafter, the sugar efflux represents the sugar phloem unloading of the grape berry. The results shown are the mean values (n = 3 replicate berry-cups) of sugar efflux (μg g^{-1} berry) from the peeled berry into the buffer solution.

concentration curve (Fig. 2). Thus, for reliable results, collection times of 20 or 30 min were chosen depending on the individual experiment.

Efflux kinetics of sugars from an in vivo *grape berry*

It has been shown that glucose and fructose were the major sugars in the phloem unloading solution. It has also been shown previously that the sugar unloaded from the phloem is accumulated in the vacuole via the apoplastic system (Gifford and Thorne, 1986; Dreier *et al*., 1998). In this study, two distinct phases were identified in the time course of the efflux: a rapid decline over the first 60 min, which in some runs, showed signs of stabilizing after 60 min

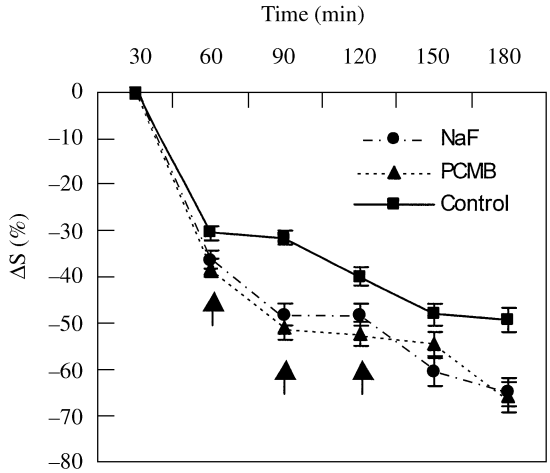

FIG. 4. Effect of NaF and PCMB on sugar unloading in grape berries. ΔS is the variation in percentage of sugar phloem unloading. Samples were taken between 1000 h and 1300 h. The arrows indicate where NaF and PCMB were included in the buffer solution. This graph shows that NaF and PCMB act rapidly in inhibiting phloem sugar unloading. The results shown are the mean values (n = 3 replicate berry-cups).

(Fig. 3). The first phase (0–60 min) is characterized by apoplastic purging and the second phase (60–160 min) is characterized by equilibration of the sugar content between the apoplast and the buffer solution. The end of the apoplastic purge was also accompanied by a transient increase in sugar efflux. As the second portion of the graph (after the arrow) shown in Fig. 3 represents phloem unloading in relation to photosynthesis (sink–source relationship), the efflux kinetics were determined in subsequent experiments after an apoplastic purge of 60 min (three buffer changes, each after 20 min).

Effect of NaF and PCMB on sugar unloading of the grape berry

Sodium fluoride (NaF) is a phosphatase inhibitor of respiratory electron transport. Incorporation of 7·5 mM NaF into the buffer solution significantly inhibits sugar phloem unloading in grape berries (Fig. 4). These results also show that NaF can act rapidly on phloem to inhibit the production of energy, and that it continues to exert this inhibitory effect for up to 60 min after it has been removed from the MES buffer solution. These findings support the observation that sugar phloem unloading is a process that requires energy in ripening grape berries (Thorne, 1985; Xia, 1999).

PCMB is a compound that modifies cysteine groups. It is lipid soluble and modifies thiol groups both at the surface and within the plasma membrane of root tissue (Wilkinson and Duncan, 1993), it has been used previously to inhibit enzyme activity (Wilkinson and Duncan, 1993; Wu *et al*., 2000).

Figure 4 shows that 2·5 mM PCMB significantly inhibits sugar phloem unloading in the grape berry, and that it continues to exert this inhibitory effect for up to 60 min after it has been removed from the MES buffer solution. These results demonstrate that phloem sugar unloading is a process

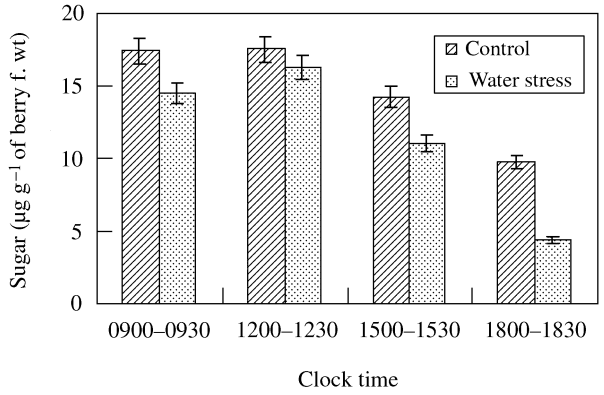

FIG. 5. Effect of water stress on sugar unloading in grape berries 82 d after anthesis. The water stress applied to the vine appears to inhibit sugar phloem unloading in the grape berry during the day mainly after 1300 h. in relation with a significant reduction of photosynthesis activity. The results shown are the mean values (n = 3 replicate berry-cups) of sugar efflux (μg g^{-1} berry) from the peeled berry into the buffer solution.

that involves a sugar transporter and the intervention of enzymes (Fillon *et al*., 1999; Patrick and Offler, 1996). The inhibition of the sugar transporter produced by PCMB was not reversed by immersion in fresh MES buffer solution for 60 min.

Effect of water stress on phloem sugar unloading

Water stress can affect the rate of photosynthesis of plant, stomatal conductance, abscisic acid content and osmotic potential; it can also affect photoassimilate translocation (Rodrigues *et al*., 1993; Winkel and Rambal, 1993; Clifford, 1998; Flexas *et al*., 1998, 1999; Yakushiji, 1998; Escalona *et al*., 1999; Kobashi, 2000; Peterlunger *et al*., 2000). During maturation, moderate water stress retards secondary shoot growth without notably affecting photosynthetic activity (Carbonneau and Deloire, 2001), thus favouring the redistribution sugar in the berries and the perennial organs. However, severe water stress can actually inhibit the accumulation of sugar in grape berries. Sugar phloem unloading is the first step in the accumulation of sugar in the sink organ (Thorne, 1985; Oparka, 1990; Patrick, 1990, 1997). Figure 5 shows that the berries of vines subjected to water stress (equivalent to approx. $\Psi_b \approx -0{\cdot}5$ MPa) accumulated less sugar than the control vines ($\Psi_b \approx -0{\cdot}2$ MPa); this effect was observed principally after 1300 h. For any given berry volume, the sugar concentration in grapes subjected to water stress is less than that of the control. This finding concurs with earlier observations relating to the effect of water stress on vine photosynthesis (data not shown), and further demonstrates the suitability of the berry-cup system to study the effects of environmental factors or chemical inhibition on the accumulation of sugar in ripening grape berries.

CONCLUSION

The berry-cup *in vivo* experimental system is a useful technique to study sugar phloem unloading and sugar accumulation in ripening grape berries. The content of sugar in the buffer solution is a realistic measure of phloem unloading following 60 min apoplastic purging. Thereafter, the buffer solution only becomes saturated with sugar after approx. 50 min. Thus, the optimum time period between buffer changes was taken to be 20 or 30 min (depending on the type of experiment). This experimental system can be used not only to study the mechanism of the sugar phloem unloading but also to study the effect of environmental factors or chemical inhibition on the accumulation of sugar in ripening grape berries. Sugar phloem unloading in ripening grape berries is shown to be via the apoplastic network and that the process requires a sugar transporter, the intervention of enzymes and a source of energy. This berry-cup technique is currently being used to investigate sugar unloading in relation to both the hydric status of the vine (water supply) and to the interception of sunlight (water demand).

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